Original Article

Full title:

The additive impact of periodic limb movements during sleep on inflammation in obstructive sleep apnea patients

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Contribution:

KM contributed to the study design, collection of data, analysis and interpretation of data and writing the manuscript. KC contributed to the study design, collection of data and editing the draft. MM contributed to study supervision. All other authors contributed to the collection of data.

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Abstract

Rationale: Both periodic limb movements during sleep (PLMS) and obstructive sleep apnea (OSA) are major causes of sleep disorders and have been associated with systemic inflammation and cardiovascular events. However, it is uncertain whether in combination they promote a higher inflammatory response and greater risk of cardiovascular events than each condition alone.

Objectives: To investigate whether the presence of PLMS is associated with increased inflammation in patients suspected of having OSA.

Methods: In 342 patients who underwent polysomnography to diagnose OSA, plasma C-reactive protein (CRP) and fibrinogen levels were measured.

Measurements and Main Results: OSA was found in 254 patients, with 46 also having PLMS. Among the 88 patients who did not have OSA, 8 had PLMS. Plasma CRP and fibrinogen levels in the group with both PLMS and OSA were higher than in patients with neither OSA nor PLMS and in patients with OSA only (CRP: 0.20±0.48 vs. 0.09±0.15 vs. 0.13±0.18 mg/dl, p=0.03; fibrinogen: 298.2±76.1 vs. 269.0±57.1 vs. 270.0±52.6 mg/dl, p <0.01) Multivariate analysis showed that the presence of PLMS was associated with higher plasma CRP levels (β=0.1401, p<0.01) and fibrinogen levels (β=0.1359, p=0.01) independently from other clinical variables such as body mass index and the severity of OSA.

Conclusions: PLMS were positively associated with plasma CRP and fibrinogen levels in patients suspected of having OSA. Since plasma levels of these proteins have been established as predictive factors of future cardiovascular events, the presence of PLMS

may be a useful clinical sign to identify OSA patients at high risk of cardiovascular events.

(253 words)

Introduction

Periodic limb movements during sleep (PLMS) are involuntary, repetitive, stereotypic, short-lasting, segmental movements of the lower and sometimes upper extremities. They occur in 5-8% of the general population and prevalence increases with age (1, 2). PLMS are identified in the vast majority of patients with restless leg syndrome (RLS), and both PLMS and RLS were reported to be associated with cardiovascular disease (CVD) and mortality (3-8). Although the causal relationship between PLMS and CVD remains uncertain, an association between PLMS and systemic inflammation has been shown, and this relationship is considered to be a factor in the increased risk of CVD in patients with PLMS (9-11). In addition, obstructive sleep apnea (OSA) syndrome is a highly prevalent sleep disorder, affecting about 4-20% of adults (12-14). OSA is characterized by repetitive episodes of partial or complete obstruction of the upper airway during sleep associated with transient oxygen desaturation. Accumulating clinical evidence suggests that OSA is an independent risk factor for CVD through impaired endothelial dysfunction and increased platelet aggregability caused by nocturnal intermittent hypoxia and subsequent chronic inflammation (15-17).

PLMS are commonly seen during polysomnography (PSG) in OSA patients, and their prevalence in OSA patients has been reported to be significantly higher than in the general population (18-20). The underlying mechanisms for the association of OSA with PLMS have not been fully elucidated nor has it been investigated whether the coexistence of OSA and PLMS promotes a greater inflammatory response than the presence of either one alone. Therefore, we hypothesized that patients with both OSA

and PLMS would have a higher inflammatory response than patients with OSA only and that comorbid PLMS is an independent risk factor for a high inflammatory response in patients suspected of having OSA. Since we have routinely measured plasma levels of inflammatory proteins such as C-reactive protein (CRP) and fibrinogen in patients in our sleep laboratory to assess patients' general condition (21), we attempted to verify these hypotheses by evaluating the data accumulated in our clinical practice.

Methods

Subjects

We examined data on all patients who underwent a diagnostic full overnight PSG at the sleep unit of Kyoto University Hospital between 2008 and 2011. All had been referred to our sleep unit under suspicion of OSA with symptoms such as habitual snoring or daytime sleepiness. Data were systematically extracted by a single investigator (KM) from patients' clinical records and PSG reports, after which they were entered into a software database for later analysis. This study protocol was approved by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee.

For a patient's data to be included in the analysis, the patient had to meet the following criteria: 1) age at least 30 years and less than 80 years and 2) no prior treatment for OSA and/or PLMS. Patients with diseases that have been reported to cause RLS and PLMS were excluded. Specifically, data on patients with Parkinson's disease, collagen diseases,

renal failure (serum creatinine level >1.3 mg/dl), anemia (hemoglobin level <12 g/dl), severe intervertebral hernia, pregnancy and with any history of heart or cerebrovascular diseases were excluded. Patients with malignancy and acute and/or chronic infection were also excluded from analysis because these conditions could possibly affect the inflammatory protein levels. Lastly, patients who were regularly taking any antidepressant, anxiolytic, anticoagulant and anti-inflammatory medications were also excluded because these medications might change the patient's PLMS status and plasma CRP and fibrinogen levels.

The definitions of the comorbid diseases are shown in the online supplement.

Procedures

Polysomnography

The diagnoses of OSA and PLMS were confirmed by PSG (SomnoStar pro, Cardinal Health, Dublin, OH, USA or Alice 4, Philips Respironics, Inc., Murrysville, PA, USA), which started at 22:00 and ended at 6:00 the following morning. A detailed description of materials and methods used for performance of polysomography is provided in the online supplement.

In the present study, the PSG studies were scored by four certified sleep laboratory technicians. To assess intra- and inter-scorer agreement, we randomly selected 20 patients (Apnea Hypopnea Index (AHI): 24.0 ± 17.4/h, PLMS index: 17.0 ± 27.1/h) from the cohort whose PLMS index was not zero. Then, intra-class correlation

coefficient (ICC) values for AHI and PLMS index scored by these technicians were calculated. The ICC values for intra-scorer agreement were more than 0.99 for AHI and 0.96 for PLMS index. The ICC value representing inter-scorer agreement was 0.98 for AHI and 0.88 for PLMS index. Because a high level of agreement for AHI and PLMS index among the technicians was found, we adopted the values for AHI and PLMS index scored by one of these four technicians for the statistical analysis in the present study. We defined AHI ≥15/h as 'OSA positive' and a PLMS index ≥15/h as 'PLMS positive' according to a previous study (1).

Anthropometric measurements were performed in the evening before PSG. In the morning following PSG, blood pressure (BP) was measured five times at one-minute intervals with the patient in the sitting position after resting for at least five minutes. The average of the latter two recordings was calculated.

Blood sampling and measurement of plasma fibrinogen level

Since OSA has been reported to be associated with various diseases such as metabolic syndrome and CVD, we have routinely recommended that patients undergo a blood test to check their status for diabetes, dyslipidemia and hypercoagulation. If patients consented, blood samples were drawn at 7:00 in the morning following a 12-h overnight fast and PSG. Thrombocheck Fib (L) (Sysmex Corporation, Kobe, Japan) is a liquid type reagent for use with the Clauss method and was employed for fibrinogen measurement. Measurements were performed using a fully automated coagulation analyzer (Coagrex 800, Shimazu Corporation, Kyoto, Japan). Aside from the plasma

fibrinogen level, we simultaneously measured blood counts, biochemistry, CRP levels and indexes of metabolic syndrome such as HbA1c and cholesterol levels.

Statistical Analysis

First, we categorized the patients into four groups according to the presence and/or absence of OSA and PLMS: “neither OSA nor PLMS’, ‘PLMS only’, ‘OSA only’ and ‘both PLMS and OSA’. The significance of intergroup differences in patients’ background was determined by an analysis of variance. Because the number of patients in the PLMS-only group was too small (n=8), that group was excluded from this intergroup analysis. When a significant difference was found, we used the Tukey’s honestly significant difference procedure to identify where the difference was significant. A chi-square test was used to compare categorical variables. Second, we used Pearson’s coefficient test to evaluate the relationships between plasma CRP or fibrinogen levels and other clinical variables for the entire cohort. In this analysis, the dichotomous variables were converted to dummy variables (‘Male’=0, ‘Female’=1 and ‘PLMS negative’=0, ‘PLMS positive’=1).

Based on results of this analysis, multivariate regression analyses were performed to clarify the contribution rate of PLMS, OSA and other comorbidities to CRP and fibrinogen levels. The variables entered into the multivariate analysis were those yielding a p value <0.10 by univariate analysis, and when two independent variables had strong collinearity ($\gamma > 0.7$), one was selected. Third, we performed the same analyses only for the cohort that was OSA positive. Data were expressed as means ±

standard deviation. Two-tailed p-values <0.05 were considered statistically significant. All statistical analyses were performed using JMP 7.0.2 statistical software (SAS Institute Inc., Cary, NC, USA).

Results

Of 841 eligible patients, 471 patients were excluded from the analysis and blood tests were not undertaken in 28 patients. Therefore, 342 patients were enrolled in the analysis (Figure 1). OSA was found in 254 patients, with 46 having PLMS. Among the 88 patients who did not have OSA, PLMS was found in just 8 patients. The prevalence rate of PLMS in the OSA-positive cohort was significantly higher than that in the OSA-negative cohort (46/254 (18.1%) vs. 8/88 (9.1%), p=0.04). Tables 1 and 2 show the clinical backgrounds of the study patients and their sleep parameters, respectively. Compared to patients with OSA only, patients with both PLMS and OSA were older and had a lower body mass index, lower diastolic blood pressure, lower hemoglobin level and milder OSA. Plasma CRP and fibrinogen levels in the group with both PLMS and OSA were higher than in patients with neither OSA nor PLMS and in patients with OSA only (CRP: 0.20±0.48 vs. 0.09±0.15 vs. 0.13±0.18 mg/dl p=0.03; fibrinogen: 298.2±76.1 vs. 269.0±57.1 vs. 270.0±52.6 mg/dl, p <0.01).

All patients had three or more hours of total sleep time (TST) during PSG recording. While TST in patients with only OSA was significantly longer than that in patients with both PLMS and OSA (390.8±74.6 vs. 363.9±78.1m, p=0.03), the indexes of severity of OSA such as the AHI and 3% oxygen desaturation index (ODI) in patients with only

OSA were significantly higher than those in patients with PLMS and OSA (AHI: 40.8±19.9 vs. 33.7±15.1/h, p=0.02, 3% ODI: 38.2±21.2 vs. 31.0±19.0/h, p=0.04) (Table 2).

Table 3 shows results of univariate and multivariate analyses of plasma CRP and fibrinogen levels for the entire cohort. Strong collinearities were found between the AHI and 3% ODI (γ=0.97) and between the PLMS index and being PLMS positive (γ=0.76). For the multiple regression analysis, we chose 3%ODI and being PLMS positive as the representative variable for OSA and PLMS severity, respectively, as these had better correlation with the CRP or fibrinogen levels in the simple correlation analysis (Table E1 in online supplement). The multivariate analyses demonstrated that being PLMS-positive was associated with plasma CRP or fibrinogen levels (CRP: β=0.1401, p<0.01; fibrinogen: β=0.1359, p=0.01) independently of other clinical variables such as body mass index (BMI) and HbA1c.

Next, as we previously noted, we performed the same analyses for the OSA-positive cohort. Table 4 shows the results of these analyses. In the OSA-positive cohort also, being PLMS positive was associated with plasma CRP or fibrinogen levels independently of clinical variables. (CRP: β=0.1466, p=0.0192; fibrinogen: β=0.1844, p=0.0036)

Discussion

The results of the present cross-sectional study indicated that patients with both OSA and PLMS had the highest plasma CRP or fibrinogen levels of the four cohorts with

suspected OSA. Furthermore, multivariate analysis showed that the presence of PLMS contributed, although weakly, to elevated plasma CRP and fibrinogen levels independently of other clinical variables. Both CRP and fibrinogen are known as acute phase proteins involved in inflammation, and elevated plasma levels of these proteins were reported to be independent risk factors for future CVD events through several mechanisms, such as a contribution to platelet aggregation, promotion of fibrin formation and increase in plasma viscosity (22-26). Therefore, the results of the present study suggested that high CRP and fibrinogen levels in patients with PLMS could be a key in clarifying why PLMS are associated with CVD. In addition, the results of this study suggest that PLMS can be a useful clinical sign to identify OSA patients at high risk of CVD. In patients with both PLMS and OSA, plasma CRP and fibrinogen levels were about 0.07 mg/dl and 30 mg/dl higher, respectively, than in patients with OSA only. Based on a previous meta-analysis that investigated the impact of elevated inflammatory protein levels on CVD, patients with both PLMS and OSA could be estimated to be 1.1-1.3 times more likely to develop CVD events than patients with OSA only (24, 27). The multivariate analysis for the entire cohort showed a significant association between the BMI and CRP level and between HbA1c and fibrinogen levels. Some previous studies showed similar relationships between these factors (28-30).

Underlying mechanism for the relationship between PLMS and the inflammatory status

Even though the results of this study did not confirm a causal relationship between

PLMS and high plasma inflammatory protein levels, several mechanisms may explain a relationship between them. Pennestri et al. reported that PLMS, whether or not associated with arousals, were correlated with repetitive nocturnal BP increments (31). Based on indications by results of some previous studies that plasma levels of inflammatory proteins were significantly associated with BP variability (32-34), BP surges provoked by PLMS may cause a heightened inflammatory status. However, in the present study, the patients with both PLMS and OSA had lower morning DBP than those with OSA only. As a possible explanation for this apparently contradictory finding, it was previously demonstrated that in OSA patients BP while awake did not always reflect BP surges during sleep (35, 36). Furthermore, the BP response to OSA and PLMS events appeared to vary depending on age (31, 37). An investigation including nocturnal BP monitoring in age-matched cohorts would help in clarifying the underlying mechanism.

In addition, as another mechanism, physical inactivity is a possible intermediary between PLMS and a heightened inflammatory state. Physical activity was reported to be inversely correlated with RLS severity and exercise was reported to decrease RLS/PLMS severity (38-40). Blood inflammatory protein levels were also reported to be inversely associated with physical activity (41-43). Furthermore, it is possible that other undetected common clinical conditions might also be responsible for an association between PLMS and elevated inflammation. According to our literature survey, the association between fibrinogen and PLMS has never been investigated in OSA patients nor in the general population. The precise mechanisms for the association between PLMS and an enhanced inflammatory response remain to be elucidated.

Relationship between OSA and PLMS

Previous studies showed that PLMS are more common in patients with sleep disordered breathing than in the general population (18, 19). In fact, in the present cohort, the prevalence rate of PLMS in OSA-positive patients was significantly higher than that in non-OSA patients. However, the mechanisms for the associations between PLMS genesis and OSA also remain to be elucidated. One possible mechanism is through obesity and dysfunction of the dopaminergic pathway. Obesity is a major risk factor for OSA, and obese people had lower dopamine D2 receptor availability in their brain striatum than normal weight individuals (44). Because a dysfunctional dopaminergic pathway is involved in the genesis of RLS or PLMS (45), OSA and PLMS could be connected with each other. However, Manconi et al. reported that dopamine agonists do not decrease the number of PLMS-associated sleep disordered breathing episodes and suggested that primary dopaminergic dysfunction may not play a major role in the relationship between OSA and PLMS (46). In fact, patients with both PLMS and OSA had a lower BMI than those with OSA only in the present study. The proposed mechanism for PLMS genesis through obesity and dopaminergic dysfunction may not be applicable in the present cohort.

The direct causal relation between PLMS and OSA is poorly understood. Exar et al. performed PSG in subjects with PLMS and monitored intrathoracic pressure during sleep by a pressure transducer catheter that was transnasally placed in the esophagus. They demonstrated that PLMS may occur in association with subtle hypopneic episodes and episodes of increased upper airway resistance that could not be identified by

conventional PSG without a transnasally placed pressure transducer (47). In addition, others indicated that moderate to severe OSA masks PLMS, which may be more fully manifested during OSA treatment because of the amelioration of frank apneas to respiratory effort-related arousals (48, 49). In contrast, other studies indicated that the severity of PLMS had decreased with treatment of OSA (50, 51). The precise mechanism for the associations among PLMS, OSA and treatment of OSA has not been elucidated.

Most previous studies that investigated the relationship between OSA and inflammatory protein levels did not include PLMS as a confounding factor (52, 53). Because the present study showed that the contribution rate of PLMS to inflammatory protein levels was similar or larger than that of OSA, taking the contribution of PLMS into consideration might lead to a more precise evaluation of the relationships between sleep disorders and inflammatory response.

Limitations

We recognized several limitations in the present study. First, the subjects were only those under suspicion of OSA. Therefore, it is likely that if the initial cohort had been from the general population a greater number of patients with PLMS might have been identified. However, with the current study design, the number of patients identified with PLMS only was too small to perform meaningful statistical analyses. Whether we can extrapolate the present results to the general population should be examined in further studies. In addition, all of the subjects in this study were Japanese. Since the

clinical characteristics of patients with OSA and/or PLMS did vary depending on their ethnicity (54, 55), whether our findings can be applied to a cohort comprised of different races should be investigated. Second, we did not evaluate the symptoms of RLS, such as dysesthesias and unpleasant sensations in the legs. Therefore, we could not identify the precise prevalence of RLS in the present cohort. Third, iron deficiency anemia is also considered as a possible cause of PLMS and RLS (56-58). In fact, in the present study the patients with both PLMS and OSA had lower hemoglobin levels than those with OSA only. Because of the retrospective design, we could not evaluate serum iron and ferritin levels. These results might lead us to undertake more sophisticated evaluations of the associations of PLMS, OSA and inflammatory protein levels. Fourth, we did not exclude patients with components of metabolic syndrome such as diabetes and dyslipidemia in order to reflect the situation encountered in actual clinical practice. In addition, to exclude patients with severe renal failure, we chose the serum creatinine level as the index of renal function in this study because values of the estimated glomerular filtration rate vary significantly depending on which predictive formula is adopted (59). The precise evaluation of renal function is of clinical concern and it was possible that this cohort included individuals with moderate renal failure. Although the presence of these comorbid diseases could possibly affect the results, we believe that the possibility was minimized because we took these factors into consideration in the statistical analysis. Lastly, this was a cross-sectional study, and we did not have data on levels of inflammatory proteins after treatment for OSA and/or PLMS. A longitudinal investigation may clarify the more precise underlying mechanisms among OSA, PLMS and an elevated inflammatory response.

Conclusion

In summary, the present study provides the first clinical evidence demonstrating that PLMS were positively associated with plasma CRP and fibrinogen levels in patients under suspicion of OSA. Because the levels of these proteins are established predictive factors of future CVD events, PLMS can be a useful clinical sign to identify OSA patients at high risk for CVD. Since the precise pathophysiologic mechanisms among OSA, PLMS and an elevated inflammatory response remain to be elucidated, further studies are warranted. Moreover, whether we can extrapolate these results to the general population should be examined in further studies to clarify the reasons why PLMS are associated with CVD.

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Table 1. Clinical backgrounds of study patients.

	Neither OSA nor PLMS (n=80)	OSA only (n=208)	PLMS only (n=8)	Both PLMS and OSA (n=46)	p*
Clinical background					
Age (y)	51.9±13.0	55.7±12.6[a]	62.9±9.6	66.1±8.5[a,b]	<0.0001
Female, n(%)	37 (46.3%)	46 (22.1%)	7 (87.5%)	13 (28.3%)	0.0004
Body mass index (kg/m^2)	25.2±4.1	27.7±5.9[a]	22.9±4.8	25.6±4.6[b]	0.0005
Brinkman index	185.2±423.9	282.8±366.0	75.0±116.5	377.6±622.3[a]	0.0439
Hypertension, n(%)	25 (31.3%)	108 (51.9%)	2 (25.0%)	26 (56.5%)	0.0027
Diabetes, n(%)	11 (13.8%)	51 (24.5%)	1 (12.5%)	14 (30.4%)	0.0514
Dyslipidemia, n(%)	45 (56.3%)	95 (45.7%)	5 (62.5%)	18 (39.1%)	0.1331
SBP (mmHg)	119.7±15.9	128.4±15.2[a]	120.6±16.3	125.4±15.7	0.0001
DBP (mmHg)	73.7±11.7	80.1±11.0[a]	69.0±12.1	75.5±11.0[b]	<0.0001
Laboratory profiles					
Hemoglobin (g/dl)	14.3±1.6	14.6±1.6[a]	13.2±1.0	14.0±1.3[b]	0.0160
Creatinine (mg/dl)	0.73±0.16	0.79±0.17[a]	0.66±0.15	0.79±0.18	0.0229
HbA1c (%)	5.4±0.9	5.8±1.1[a]	5.5±0.6	5.8±0.8[a]	0.0079
Total protein (g/dl)	6.8±0.5	6.7±0.4	6.7±0.3	6.8±0.4	0.4431
LDL cholesterol (mg/dl)	112.9±27.0	115.7±32.1	116.0±33.2	114.0±23.4	0.7662
HDL cholesterol (mg/dl)	52.6±13.9	49.8±12.3	56.5±6.9	52.7±16.4	0.1760
Triglyceride (mg/dl)	133.2±89.0	143.2±85.2	91.8±22.8	122.2±59.1	0.2530
C-reactive protein (mg/dl)	0.09±0.15	0.13±0.18	0.14±0.25	0.20±0.48[a]	0.0259
Fibrinogen (mg/dl)	269.0±57.1	270.0±52.6	289.6±56.5	298.2±76.1[a,b]	0.0082

Data are expressed in mean ± SD. PLMS: periodic limb movements during sleep; OSA: obstructive sleep apnea; SBP: systolic blood pressure; DBP: diastolic blood pressure; LDL: low-density lipoprotein; HDL: high-density lipoprotein.
* p value determined by analysis of variance among groups of patients.

However, the PLMS-only group was excluded from this inter-group analysis because of the small number of patients.

When a significant difference was found among three groups, post hoc analysis was performed to identify where the difference was significant. [a]: $p<0.05$ vs. Neither OSA nor PLMS, [b]:$p<0.05$ vs. OSA only.

Table 2. Sleep parameters of study patients

Data are expressed in mean ± SD. OSA: obstructive sleep apnea, PLMS: periodic limb movements during

	Neither OSA or PLMS (n=80)	OSA only (n=208)	PLMS only (n=8)	Both PLMS and OSA (n=46)	p*
Time in bed (m)	529.1±53.1	526.0±53.1	503.6±46.8	538.4±48.2	0.3442
Total sleep time (m)	420.2±67.2	390.8±74.6[a]	410.1±81.1	363.9±78.1[a,b]	0.0001
AHI (/h)	7.9±4.3	40.8±19.9[a]	7.9±4.0	33.7±15.1[a,b]	<0.0001
3%ODI (/h)	5.8±4.4	38.2±21.2[a]	5.9±4.7	31.0±19.0[a,b]	<0.0001
Apnea index (/h)	1.9±2.5	20.6±18.4[a]	2.0±1.1	15.4±14.0[a]	<0.0001
SpO_2 <90% time (m)	4.5±10.0	94.8±120.7[a]	0.7±0.8	61.6±103.4[a]	<0.0001
Minimum SpO_2 (%)	88.4±4.6	76.9±10.9[a]	89.4±5.6	78.1±10.0[a]	<0.0001
Non slow wave sleep (%)	73.8±9.1	80.5±9.9[a]	82.3±4.3	81.7±7.9[a]	<0.0001
Slow wave sleep (%)	7.8±7.5	4.8±7.1[a]	2.3±4.1	4.5±5.5[a]	0.0028
REM sleep (%)	18.4±5.7	14.7±6.1[a]	15.4±2.8	13.8±5.3[a]	<0.0001
PLMS index (/h)	1.5±3.5	1.4±3.3	36.1±14.9	41.9±31.9[a,b]	<0.0001
PLMS with arousal index (/h)	0.2±0.6	0.2±0.8	5.2±4.8	3.5±5.5[a,b]	<0.0001

sleep; AHI: apnea hypopnea index; ODI: oxygen desaturation index; REM; rapid eye movements.

*: p value determined by analysis of variance among groups of patients. However, the PLMS-only group was excluded from the intergroup analysis because of the small number of patients.

When a significant difference was found among three groups, post hoc analysis was performed to identify where the difference was significant. [a]: $p<0.05$ vs. Neither OSA nor PLMS, [b]:$p<0.05$ vs. OSA only.

Table 3. Univariate and multivariate regression analyses for the entire cohort (n=342) using the C-reactive protein or fibrinogen level as the dependent variables

	CRP				Fibrinogen			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	r	p	β	p	r	p	β	p
Age (y)	0.0223	0.6808	-	-	0.1703	0.0016	-	0.2848
Female	<0.001	0.9841	-	-	0.1364	0.0115	-	0.2904
Body mass index (kg/m^2)	0.2191	<0.0001	0.2090	0.0005	0.1229	0.0229	-	0.2738
Brinkman index	0.1034	0.0562	-	0.0629	0.1673	0.3219	-	-
SBP (mmHg)	0.1034	0.0563	-	0.5497	0.1323	0.0141	-	0.1142
Hemoglobin (g/dl)	<0.0001	0.8770	-	-	-0.1786	0.0009	-	0.0610
Creatinine (mg/dl)	<0.0001	0.8670	-	-	-0.0837	0.1446	-	-
HbA1c (%)	0.1170	0.0306	-	0.4262	0.1729	0.0014	0.1181	0.0296
Total sleep time (m)	0.0282	0.5933	-	-	-0.2358	0.1831	-	-
3%ODI (/h)	0.1526	0.0047	-	0.4284	0.1442	0.0076	-	0.0646
PLMS positive	0.1183	0.0291	0.1401	0.0085	0.1712	0.0015	0.1359	0.0128

r: correlation efficient; β: standard regression coefficient; SBP: systolic blood pressure; ODI: oxygen desaturation index; PLMS: periodic limb movements during sleep.

Table 4. Univariate and multivariate regression analyses for the OSA cohort (n=254) using the plasma C-reactive protein or fibrinogen level as the dependent variables.

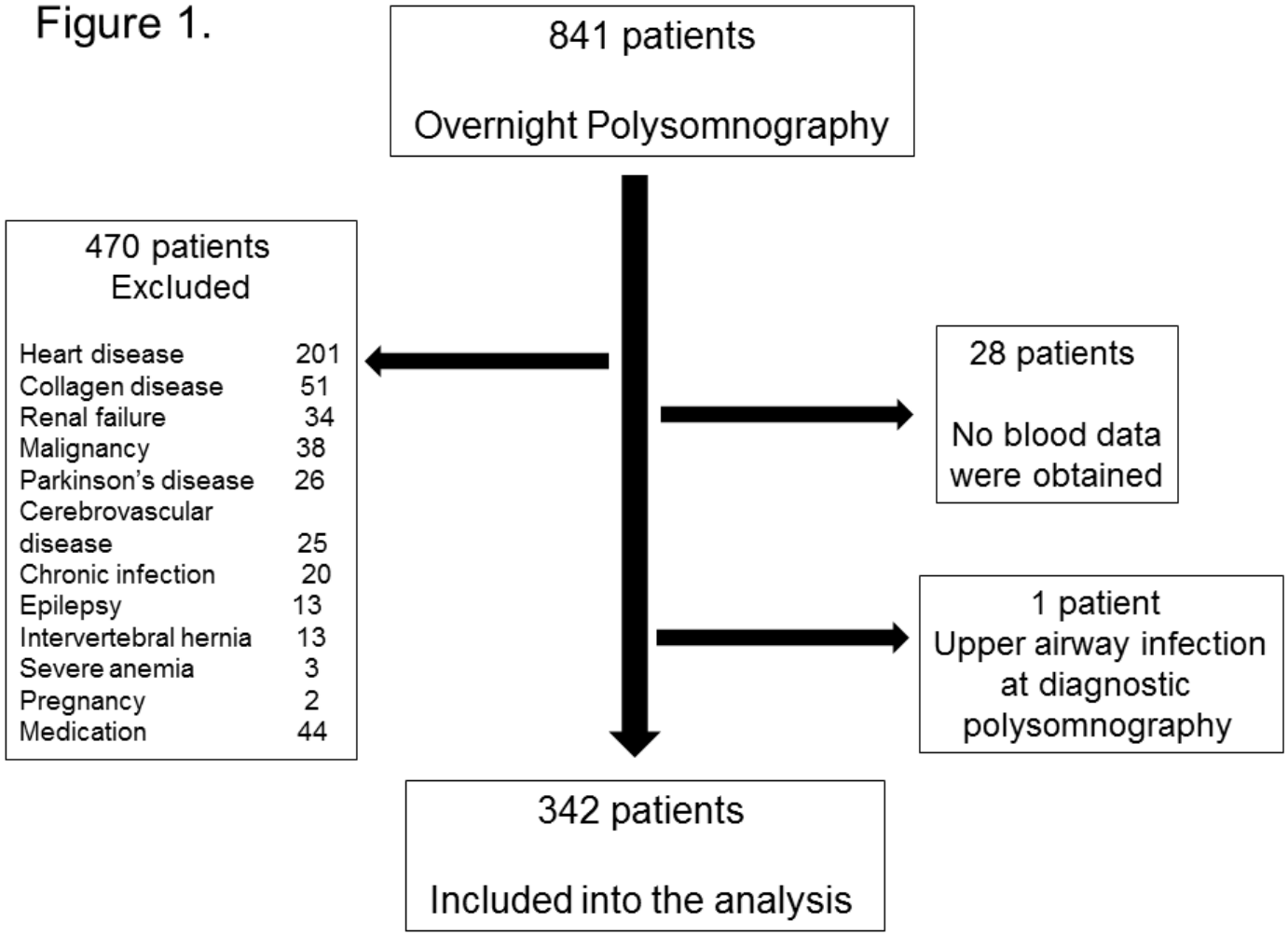
	CRP				Fibrinogen			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	r	p	β	p	r	p	β	p
Age (y)	0.0400	0.5264	-	-	0.1345	0.0322	-	0.5280
Female	0.0420	0.5047	-	-	0.1507	0.0162	-	0.5029
Body mass index (kg/m^2)	0.1863	0.0029	0.1794	0.0086	0.1414	0.0242	-	0.4011
Brinkman index	0.1000	0.1109	-	-	<0.0001	0.8851	-	-
SBP (mmHg)	0.1034	0.1006	-	-	0.1432	0.0223	-	0.0815
Hemoglobin (g/dl)	<0.0001	0.9118	-	-	-0.2020	0.0012	-	0.0530
Creatinine (mg/dl)	0.0678	0.2839	-	-	-0.1030	0.1009	-	-
HbA1c (%)	0.0520	0.4130	-	-	0.1389	0.0268	-	0.1377
Total sleep time (m)	0.0100	0.5430	-	-	-0.2156	0.3373	-	-
3%ODI (/h)	0.1225	0.0509	-	0.3183	0.1715	0.0062	0.1855	0.0071
PLMS positive	0.1118	0.0749	0.1466	0.0192	0.1860	0.0029	0.1844	0.0036

r: correlation efficient; β: standard regression coefficient; SBP: systolic blood pressure; ODI: oxygen desaturation index; PLMS: periodic limb movements during sleep.

Figure legend

Figure 1. Flow chart of patient selection.

Figure 1.


841 patients
Overnight Polysomnography
470 patients
Excluded
Heart disease 201
Collagen disease 51
Renal failure 34
Malignancy 38
Parkinson's disease 26
Cerebrovascular disease 25
Chronic infection 20
Epilepsy 13
Intervertebral hernia 13
Severe anemia 3
Pregnancy 2
Medication 44
28 patients
No blood data were obtained
1 patient
Upper airway infection at diagnostic polysomnography
342 patients
Included into the analysis

ONLINE DATA SUPPLEMENT

The additive impact of periodic limb movements during sleep on inflammation in obstructive sleep apnea patients

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Methods

The definition of comorbid diseases

Hypertension was defined by a systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg or previous treatment for hypertension. Diabetes mellitus was defined by HbA1c ≥6.0% or previous treatment. Dyslipidemia was defined by triglycerides ≥150 mg/dl, high-density lipoprotein cholesterol level <40 mg/dl, low-density lipoprotein cholesterol level ≥140 mg/dl or specific treatment for these lipid abnormalities. Smoking status was evaluated by the Brinkmann index, which represents the number of cigarettes smoked per day multiplied by the number of years of smoking.

Polysomnography

The diagnoses of obstructive sleep apnea (OSA) and periodic limb movements during sleep (PLMS) were confirmed by polysomnography (PSG) (SomnoStar pro, Cardinal Health, Dublin, OH, USA or Alice 4, Philips Respironics, Inc., Murrysville, PA, USA), which started at 22:00 and ended at 6:00 the following morning. Surface electrodes were attached using standard techniques to obtain an electrooculogram, electromyogram (EMG) of the chin and 12-lead electroencephalogram (EEG). Sleep stages were defined according to the criteria of Rechtchaffen and Kales.(1) Ventilation was monitored by inductive plethysmography (Respitrace QDC, Viasys Healthcare, Palm Springs, CA, USA). Airflow was monitored by a nasal pressure transducer and supplemented by an oronasal thermal sensor. Arterial oxygen saturation (SpO_2) was monitored continuously with a pulse oximeter. Apnea was defined as the complete cessation of airflow and hypopnea as a clear decrease in airflow of 50% lasting more than 10 s and followed by

either a decrease in SpO_2 of at least 3% or EEG arousal.(2) All apnea hypopnea index (AHI) values were expressed as the number of episodes of apnea and hypopnea per hour over the total sleep time. 3% oxygen desaturation index (ODI) values were defined as the number of desaturations ≥3% per hour of sleep. The length of time of SpO_2<90% during sleep was calculated in each patient.

All movements of the left and right legs were recorded independently from the anterior tibialis EMG using surface electrodes. We scored PLMS based on the standard American Academy of Sleep Medicine criteria in which individual movements were scored as PLMS if the duration was between 0.5 and 5 s and when there was a clear increase in amplitude from baseline in leg channels.(3) To be considered periodic, at least 4 movements needed to occur in succession no less than 5 s and no more than 90 s apart. Leg movements that occurred at resolution of an apnea or hypopnea were not scored as PLMS. The PLMS index was the total number of periodic leg movements per hour of sleep. The PLMS arousal index was determined as the total number of periodic leg movements per hour of sleep in which EEG arousal occurred within 1 s of movement termination.

Blood sampling and measurement of plasma fibrinogen level

Blood samples were drawn at 7:00 in the morning following a 12-h overnight fast and PSG. Thrombocheck Fib (L) (Sysmex Corporation, Kobe, Japan) is a liquid type reagent for use with the Clauss method and was employed for fibrinogen measurement. Measurements were performed using a fully automated coagulation analyzer (Coagrex 800, Shimazu Corporation, Kyoto, Japan). The intra- and inter-assay coefficients of variation for this method of measurement were less than 15% and 6%, respectively.

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Table E1. The correlation coefficients between plasma inflammatory protein levels and indexes of obstructive sleep apnea or PLMS.

(A)

	r	p
Apnea Hypopnea Index, /h	0.1449	0.0073
3% oxygen desaturation index, /h	0.1526	0.0045
PLMS index, /h	0.0656	0.2236
PLMS positive	0.1187	0.0284

(B)

	r	p
Apnea Hypopnea Index, /h	0.1315	0.0149
3% oxygen desaturation index, /h	0.1442	0.0076
PLMS index, /h	0.1327	0.0142
PLMS positive	0.1712	0.0015

(A) Simple correlations between plasma C reactive protein levels and indexes of obstructive sleep apnea or PLMS.

(B) Simple correlations between plasma fibrinogen levels and indexes of obstructive sleep apnea or PLMS.

r: correlation coefficient; PLMS: periodic limb movements during sleep.